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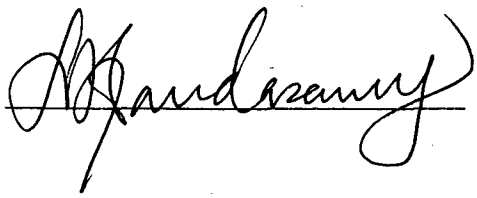

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13. ABSTRACT *(Maximum 200 Words)*

The laboratory rat is an important model for studying breast cancer due to the similarities in this disease between rats and humans. However, limited knowledge in manipulating the rat genome through transgenesis has prevented researchers from answering important questions in breast cancer research. We proposed to carry out detailed studies to optimize the variables in transgenic manipulation, to extend transgenic rat technology to inbred rat strains, and to develop rat embryo cryopreservation. We have evaluated multiple variables in the microinjection procedure and generated multiple transgenic rat lines which should be important for breast cancer research. We have developed an efficient cryopreservation procedure for rat embryos. Embryos from the outbred Sprague-Dawley and inbred Wistar-Furth and Copenhagen strains that possess unique characteristics for breast cancer research and multiple lines of transgenic rats that are useful for breast cancer research have been frozen. Lastly, due to the lack of rat embryonic stem cells that contribute to the germline, we have set up the technology for rat cloning, a technique which could allow researchers to generate germline mutations in cellular genes of the rat. This technology is critical for generating recessive mutations which are commonly described as the genetic basis for cancers.

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Anne E. Griep 12/22/99

PI - Signature Date

TABLE OF CONTENTS

Please note: Page 3 (abstract), pages 9-16, pages 21-24 and the attachments contain unpublished data and should not be distributed.

5. Introduction

5a. General Background

Breast cancer is one of the leading cause of death among women, with one out of every nine women in the United States being predicted to develop this disease during her lifetime. As with all cancers, breast cancer is a disease in which numerous cellular and molecular genetic changes are thought to contribute to the multistaged progression of normal cells to a population of cells with unrestricted growth and metastatic potential. Over the last decade two classes of genes, cellular protooncogenes and tumor suppressor genes, have been identified as genes which play critical roles in regulating cell growth and differentiation. Deregulation of gene expression through chromosomal translocation or mutation in the regulatory elements of the gene, alterations in the activities of these gene products through mutation in the coding regions of the genes, or complete loss of these genes from the chromosome through mutation are considered to be mechanisms contributing to the failure of cells to maintain normal growth characteristics.

Both mice and rats have been extensively used as laboratory animal models in breast cancer research, as well as in cancer research in general. For several reasons, the rat is perhaps the more suitable of the two with respect to a model system for human breast cancer. Whereas a high percentage of breast cancer in the mouse is associated with the integration of the mouse mammary tumor virus (MMTV) into the *int*-1 locus with consequent deregulation of *int*-1 expression, there is no known viral etiology of breast cancer in rats, as in humans (1). Second, the progressive disease that leads to breast cancer in laboratory rats bears striking histological similarity to that seen in human breast cancer (2-4). Third, a high percentage of the resulting rat mammary cancers are hormonally responsiveness, closely mimicking that seen in human breast cancer. Finally, certain inbred strains of rats show susceptibility to breast cancer whereas others show resistance (5,6). Through genetic crosses between these strains, putative suppressors have been identified (7-9). This genetic susceptibility to breast cancer seen in the rat may bear similarity to the human disease where genetic predisposition is considered to be an important factor (10,11). In part for these reasons, the rat is accepted as the animal model of choice for screening chemopreventive drugs for human breast cancer therapy (2).

Transgenic mice have been widely used in breast cancer research. Mouse models have been developed in which the expression of deregulated *int*-1 (12), c-*myc* (13-16), activated H-*ras* (13, 17), activated c-*neu* (18-20), wild type c-*neu* (21), deregulated growth hormone (22), and deregulated transforming growth factor a (23-25) has occurred in mammary tissue. All cases lead to abnormalities in mammary epithelial cells ranging from epithelial cell hyperproliferation without tumor formation to tumor formation, apparently some being similar to ductal carcinoma in situ which is seen in human breast cancers. The most prevalent genetic alterations in human breast cancers appear to be amplification of the c-*neu* locus (26-28), found in approximately 20% of breast cancers, and mutations of p53 (10,11). Unfortunately, discrepancies between the phenotypes of the several activated-*neu* transgenic mouse models has resulted in the lack of a consensus as to the nature of the activities of the *neu* oncogene in mammary carcinoma in these models. A more promising result was obtained from investigators who analyzed transgenic mice with deregulated expression of the wild-type *neu* proto-oncogene in mammary tissue. These mice developed focal mammary carcinomas, but only after long

latency. The loss of p53 function through gene knockout led to only a very low percentage of animals with mammary adenocarcinoma (1 out of 26 p53 null mice) whereas there was a high incidence of malignant lymphomas (20 out of 26). These studies provide the best animal models to date for studying the correlation between disruptions in expression or activities of these cellular genes and the incidence of mammary carcinoma. However, they may not be truly reflective of the genetics, histopathology, or the progressive nature of human breast cancer.

Considering the depth of knowledge generated by previous studies of breast cancer in the rat and the striking parallels between the rat and human disease, the availability of transgenic rat technology would greatly enhance breast cancer research. Transgenic rats would provide an alternative, and perhaps more suitable, animal model for dissecting the molecular mechanisms of mammary carcinogenesis and testing putative therapeutic agents. In addition to providing good models for breast cancer, the rat has been widely used for biochemical and metabolic studies, owing to its larger size. A large portion of research in neuroanatomy and neurophysiology is based upon the rat. The rat is the animal in which the multistage nature of hepatocarcinogenesis has been established and studied (29). All these areas of research would profit immensely from the availability of transgenic rats.

Recently, the Transgenic Animal Facility at the University of Wisconsin Biotechnology Center developed the capacity to generate transgenic rats, primarily with the encouragement of two university colleagues, Dr. Henry Pitot, an expert in hepatocarcinogenesis, and Dr. Michael Gould, an expert in breast cancer. Through our initial attempts at transgenic rat production, we have successfully generated transgenic rats for each of these cancer researchers. However, the state of transgenic rat technology is rudimentary compared to that for transgenic mice and as such has received only limited use to date. Despite our initial successes, the production of transgenic rats is at present an extremely laborious task. As a consequence of the technical impediments we now encounter, the time and cost for generating transgenic rats is many fold higher than that for the generation of transgenic mice. For many investigators, this high cost is prohibitive. Thus, only with further improvements will this technology be as accessible for the generation of transgenic rats as it has been for the generation of transgenic mice.

Because we foresee a long term and expanding demand for transgenic rats, especially in the breast cancer research field, we propose an investigation designed to optimize transgenic rat production. This proposal to optimize transgenic rat technology was initiated because we believe that significant improvements can be made in both microinjection and embryo transfer techniques which would greatly facilitate transgenic rat technology. These advances should lead to the reduced cost in the production of transgenic rats, and to the capacity to generate transgenic rats in inbred backgrounds. Importantly, during the course of our optimization studies, a series of transgenic rat models for breast cancer research will be generated.

5b. Specific Aims and Statement of Work

Therefore, we proposed this infrastructure enhancement grant to provide a resource to the breast cancer research community for the generation of novel transgenic rat models for breast cancer research. The specific aims we proposed are:

(1) To generate transgenic rat lineages specifically for breast cancer research and to make these transgenic rats readily available to the breast cancer research community at a reasonable cost.

(2) To determine the most efficient technical procedures for the rapid generation of transgenic rat lineages on an outbred genetic background and on inbred genetic backgrounds appropriate for breast cancer research.

(3) To develop efficient procedures for rat embryo cryopreservation.

(4) To develop and maintain the necessary resources and establish procedures or ongoing data sharing and communication amongst transgenic rat laboratories and with breast cancer researchers.

To accomplish these specific aims, we developed a Statement of Work that incorporated aspects of all four specific aims into each of two chronological stages. Stage One dealt with the optimization of technologies for transgenic rat production and cryopreservation using outbred rat strains and Stage Two with optimization for transgenic rat production and embryo cryopreservation using inbred rat strains. The first stage of the Statement of Work, designed to cover years 1 and 2 of the grant period, included the following points:

(a) Using MMTV-*neu*^{WT} and MMTV-*neu*^{mut} as test DNAs, optimize variables in microinjection and embryo transfer in the outbred Sprague-Dawley background.

(b) Maintain a small breeding colony of the *neu* transgenic rats (6 lineages) for dissemination to other breast cancer researchers.

(c) Develop embryo cryopreservation for Sprague-Dawley rat embryos. Cryopreserve *neu* transgenic rat lineages.

(d) Solicit requests for DNAs from the breast cancer research community. Have advisory board choose DNAs, judged to be of the greatest potential value to breast cancer research, for microinjection during years 3 and 4.

(e) Develop and make available to transgenic rat and breast cancer research communities specialized information databases.

Over the five years of this grant we have made substantial progress towards making the rat a more useful organism for genetic animal models of breast cancer. We have generated multiple strains of transgenic rat that are important models for breast cancer (Specific Aim 1). We have optimized microinjection technique and cryopreservation procedures for rat embryos maintained on outbred and inbred genetic backgrounds (Specific Aims 2 and 3). Finally, we have initiated efforts to develop rat cloning as a means of providing a mechanism to generate rat knockouts (Specific Aim 2). The ensuing body of this report summarizes our work on the aims of this grant over the entire period of the grant.

6. Body

6a. Statement of Work Point A (Specific Aim 1): Using MMTV-*neu*^{wt} and MMTV*neu*^{mut} as test DNAs optimize variables in microinjection and embryo transfer in outbred Sprague-Dawley background: Generation of transgenic rats carrying MMTV-*neu*^{wt} and MMTV-*neu*^{mut} DNAs.

An essential aim of this grant is to generate new valuable transgenic rat strains for breast cancer research. During the first years of this grant, we generated numerous transgenic rat lineages with several DNAs of interest to breast cancer research. These DNAs were as follows: First, MMTV-*neu*^{wt}, which consists of a mouse mammary tumor virus long terminal repeat driving expression of the wild type neu protooncogene. The MMTV promoter sequences have been demonstrated to drive expression of linked genes to the mammary epithelium of transgenic mice (19-21) and, hence, would be expected to do so in the rat as well. The neu oncogene has been shown to be a frequently mutated gene in human breast cancers (26-28). Thus, a rat model where high levels of wild type neu would be expressed should be of value in evaluating the role of this protooncogene in breast cancer. Furthermore, such a rat model could be used in studies to evaluate the role of carcinogenic agents as cofactors in neu-associated breast cancers. The second DNA, called MT-*neu*^{mut} we chose to use is one where a mutated neu oncogene is fused to the mouse metallothionein promoter which is inducible by heavy metals such as zinc (22,23,25). The inducible approach was chosen to express the activated oncogene because of the worry that if expression of a mutated oncogene occurred too early in the life of the rat, stable rat lines would never be derived. The final DNA, called H*ras*-K*ras* consists of the transcriptional control regions of the H-*ras* gene fused to the coding sequences of the K-r*as* gene. Activated H-r*as*, but not K-*ras*, is frequently found in rat mammary carcinomas arising as a consequence of treatment with carcinogens (13,17). This transgene DNA is one of a series of transgenes designed to study the mechanisms whereby this differential activation occurs following carcinogen treatment. We had previously generated multiple transgenic rat lineages with the above DNAs. The results of studies with the MMTV-*neu*^{wt} lines in Dr. Michael Gould's laboratory in the Department of Human Oncology at University of Wisconsin indicated that generation of an additional line would be desirable. Thus, we generated recently an additional line of MMTV-*neu*^{wt} transgenic rats. Finally, during the past year we generated a series of four independent transgenic rat lines with an MMTV-IGF2Receptor transgene. This receptor has been implicated in the development of breast cancer as it is thought to be upregulated during the process. The transgenic model should mimic this state of IGF2R, allowing investigators to reveal whether or not there is a direct tie between IGF2R upregulation and specific events in mammary cancer formation. Data concerning the various transgenic rats we have generated are incorporated into Table 1 (see below).

A goal listed for this grant was to solicit requests from the breast cancer research community at large for additional DNAs to use in the generation of transgenic rat models for breast cancer research (statement of work point d). We had arranged to collaborate with Dr. Gail Sonenshein, Professor of Biochemistry at Boston University, who is studying the role of NF-kB/rel in mammary tumors. Her initial animal studies have used a mouse model, but she feels as though a rat model would be desirable. Given that her DNA construct is proven to express as expected in mouse, we anticipated success in generation valuable rat models for her work. However, her plans have changed and she has decided not to pursue the transgenic rat. Despite the fact that we were not able to generated any transgenic rat models for breast cancer for researchers outside the University of Wisconsin, we have through this effort made a large number of investigators aware that it is possible to generate transgenic rat models that mimic human

diseases. Many investigators have contacted us to discuss the possibilities of our generating transgenic rat models specific for the disease they are investigating.

6b. Statement of Work Point A (Specific Aim 2): Using MMTV-*neu*^{wt} and MMTV*neu*^{mut} as test DNAs optimize variables in microinjection and embryo transfer in outbred Sprague-Dawley background: Optimize variables in microinjection and embryo transfer technique.

The second aim of our studies is to investigate ways to increase the efficiency and ease of transgenic rat production. At the present time, it takes roughly three to four times as longer to generate a transgenic rat than it does to generate a transgenic mouse and we typically find 15-20% of our candidate mice to be transgenic. It is also considerably more costly due to both the higher costs for purchase of rats and the costs of labor time. If transgenic rats are to become as well used in cancer research as are transgenic mice, the barriers to efficient production that currently exist must be overcome. It was our aim, to systematically evaluate the components of the standard transgenic production procedures to assess which steps in the process can be modified to facilitate transgenic rat production. We considered testable several components of microinjection technique and transfer technique and over the course of the first three years of the grant period, systematically evaluated these variables. A summary of these efforts is presented in this report and in Table 2. Please refer to the annual reports from 1995 and 1996 for more detailed descriptions of these experiments.

Our data have been evaluated using mainly in vivo because ultimately, we desire to produce transgenic rats at higher efficiency than we do currently. It is possible that certain components of the medium may have little impact on embryo viability *in vitro* yet have a significant impact on embryo viability *in vivo* or on pregnancy rates. Likewise, the opposite scenario may also be true. Our criteria were the percentage of injected embryos that are lysed by microinjection, the pregnancy rate of recipients, the percentage of transferred embryos that go on to produce live births, the percentage of injected embryos that produce live births, the percentage of injected embryos that are identified as transgenic rats and the percentage of transferred embryos that are identified as transgenic rats. We used least two of the three DNAs described above for each variable to discount any possible DNA-specific effect. Because the trends were the same for each DNA, we conclude that the effects were observed were unrelated to the particular DNA and were related to the variable being tested. In addition, we routinely transfer uninjected coat-color marked carrier embryos to ensure pregnancy of our recipient rats. These carrier embryos were treated with the same media variations as were the microinjected embryos. This manipulation allowed us to distinguish between effects on embryo viability in the absence of microinjection and effects on viability associated specifically with microinjection.

Prior to the initiation of this grant period, we had used conventional M2 mouse medium for our rat microinjection experiments. Among the components in M2 medium are glucose and phosphate. Evidence from work on hamster embryos suggested that glucose and phsophate may affect the viability of rat embryos. Removal of glucose from the medium (called NG medium) used for microinjection and transfer resulted in the same percentage of embryos which lysed following microinjection. Pregnancy rates were slightly higher when medium lacking glucose was used. The percentage of injected embryos transferred into recipients which were born was slightly higher when the medium lacked glucose. Notably, the percentage of pups born that were identified as transgenic was higher, 17% versus 11%. Thus simply removing glucose from the culture medium provided a modest increase in the ultimate success rate of transgenic rat production. The effect of removal of phosphate was the opposite. Removal of phosphate

from the medium (NP) did not affect the lysis rate after microinjection but did result in decreased pregnancy rates, decreased percent of injected transferred embryos born and only 4% of pups born being transgenic. A combination of no phosphate and no glucose (called NPNG medium) resulted in no change in lysis rate, control levels of pregnancy and only 5% of rats born being transgenic. Thus, while no glucose appears to be beneficial, no phosphate appears to be detrimental. The effects of glucose are consistent with the available data on in vitro culture; however, the negative impact of phosphate removal is unexpected. It is possible that one cell embryos require phosphate, at least during the microinjection process, and that subsequent in vivo development is not adversely affected by phosphate or that dilution of the transfer medium by the oviductal fluids reduces the phosphate concentration enough so that it no longer inhibits development.

It has been reported that a reduction in the concentrations of glucose and addition of glucosamine to the culture medium resulted in less ruffling of the vitelline membrane which would make visualization of pronuclei easier (31). Glucosamine also reportedly supported faster healing of the membrane after microinjections. Thus, glucosamine treated embryos should be easier to microinject and should be less prone to lysis after microinjection. Thus, we tested the effects of glucosamine on microinjection success rates and on the rate of transgenic rats born from embryos manipulated in glucosamine (called DM2 medium). While glucosamine in our hands did result in less membrane ruffling, making microinjection easier, it only modestly improved the lysis rate (a drop from 33% to 20%). Furthermore, transferring embryos into recipients in medium containing glucosamine drastically reduced the pregnancy rate to only 22% as compared to the M2 levels of 70%. In protocols where glucosamine is used, embryos are presoaked in glucosamine then moved to normal medium for microinjection and transfer. We performed microinjections where the embryos presoaked in glucosamine were microinjected in either M2 (called DPM2 medium) or in no glucose medium (called DPNG medium). Our experience with this manipulation is that the protective effect of glucosamine only persists for about 30-45 minutes after removal from the glucosamine-containing medium. Therefore, one would need to move small groups of embryos at a time to medium suitable for microinjection and transfer. Pregnancy rates recovered to normal levels; however, the percentage of rats born from injected transferred embryos remained lower than control and the percentage of transgenics in the population of pups born was low as well. Since our goal is to minimize the numbers of manipulations through which the embryos must pass, we do not favor the use of glucosamine because the negative consequences of this manipulation outweigh the positive effects.

Finally, we evaluated the possibility that the addition of 7.5 μg/ml cytochalasin B, a microfilament inhibitor, to injection medium would facilitate microinjection (called NPNG cytoB medium). Using bovine embryos, cytochalasin B has been shown to increase the rigidity of the membranes and to restrict the movement of the pronuclei with respect to the membrane (F. Barnes and N. First, personal communication). Cytochalasin B has also been used in mouse nuclear transplantation experiments without any long term effects on the viability of the embryos. In our hands, cytochalasin B had an observable effect on stiffening the membranes and led to a large decrease in the lysis rate from 33% to 11% of microinjected embryos. It was also our experience that each embryo was easier to microinject thereby reducing the overall time required for microinjection of large group of embryos. Unfortunately, we had chosen to include the cytoB in NPNG medium rather than in NG medium which appeared to provide the highest overall percentage of transgenic rats born. Testing the success of transgenic rat production in NG medium plus cytochalasin B is our next priority. With a two-thirds reduction in the numbers of embryo lysed by microinjection combined with a modest

increase in the percentage of pups born transgenic, we expect to be able to produce transgenic rats more rapidly with greater ease.

We evaluated two aspects of transfer protocol. The first was exactly how to enter the oviduct through the bursa. The second was to determine the maximum number of embryos that one can transfer into one recipient rat before there is decreased numbers of rats born. When entering the rat oviduct, great care must be taken to avoid rupturing the large blood vessels on the bursa. One can cut the bursa, carefully avoiding these vessels, and peel it back. This maneuver generates a large opening through which to find the infundibulum of the oviduct. Alternatively, one can easily make a large hole in the bursa, avoiding the blood vessels, which enables one to more easily see the infundibulum. Either manipulation appears to have no detrimental effect on pregnancy rate. From our experiments with transferring varying numbers of embryos into a recipient, one gets optimal recovery of transferred injected embryos if between 40-50 embryos are transferred into each recipient Sprague-Dawley rat (see Fig. 1 in the annual report from 1995).

Thus, during the first few years of this grant, we made significant progress towards evaluating the technical factors that influence the ultimate success in producing transgenic rats (refer to the progress report from year 1 for details on these experimental manipulations). After numerous studies, we concluded that we could not identify any other variables that might affect microinjection efficiency that we could test. We also felt confident that variations in efficiency between different DNA constructs was inevitable and probably relates to the quality of the DNA prep, as it is for injection of mouse embryos.

6c. Statement of Work Point C (Specific Aim 3): Develop efficient methods for cryopreservation of Sprague-Dawley rat embryos.

As mentioned in background, we have established previously efficient methods for the cryopreservation of transgenic mouse embryos. It is clear that adaptation of this technology to the rat will be critical for the long term success of transgenic rat programs in our facility as well as world-wide. When we began our work to develop techniques for efficient cryopreservation of rat embryos, very little data were available in the literature; the only literature sources coming from Japanese groups who performed there studies exclusively with the Wistar strain of rats. (30, 31).

The freezing and thawing process requires that one be able to dehydrate the embryo with a cryoprotectant before freezing and then rehydrate the embryo after freezing without losing viability of the embryo. There are multiple variables that could affect embryo viability including the rate of freezing, the actual final temperature of freezing via controlled rate, the cryoprotectant used, the stage of embryo frozen. In the first few years of this grant, we determined the conditions necessary for successfully cryopreserving morulae from Sprague-Dawley rats. We additionally showed that we had determined that these procedures can be used to cryopreserve embryos from the inbred Wistar-Furth strain. We then determined that the efficient production of embryos for cryopreservation can be accomplished by superovulation regimens in which FSH and LH are administered to the female rat prior to mating (see annual reports 1996 and 1997 for detailed descrptions of these experiments and data obtained). We also reported that we had frozen and thawed hundreds of rat morulae with 90% viability which yielded approximately 35% live births when transferred into pseudopregnant recipients (Table 3).

We have determined that the superovulation and cryopreservation procedures that we have used for cryopreserving Sprague-Dawley and Wistar-Furth rats also are suitable

for freezing morulae from the Copenhagen inbred strain (Table 3). Approximately 90% of Copenhagen embryos were viable when thawed and 14% live births were recovered from embryos transferred into pseudopregnant recipients. While the percent of live births is low (about half that of Sprague-Dawley and Wistar-Furth), these results are derived from a very small sample size and therefore variables beyond our control may have affected recovery. While it is not certain that these exact conditions will be optimal for any strain, we believe that they are likely to be suitable for a wide variety of other rat strains and with limited experimentation could be optimized for other strains, if necessary.

In the past two years, we have cryopreserved various transgenic rat strains. We have cryopreserved one strain of MMTV-*neu*^{wt} transgenic rats, 3 strains of Hras-Kras transgenic rats and 5 strains of Hras-Hras transgenic rats for Dr. Gould's laboratory. Additionally, we have cryopreserved 6 congenic lines of rats on the Wistar-Furth background (Table 4). These cryopreserved stocks provide a repository of these valuable strains that permits their "immortalization" without the huge time and cost investments that are required to maintain these animals alive. Additionally, cryopreservation protects these strains against disease, loss due to catastrophic events, and genetic drift. At any time in the future, these strains can be recovered for any interested researcher in the breast cancer field.

6d. Statement of Work Stage 2 Point B and C (Specific Aim 2) Using novel DNAs provided by breast cancer researchers and adapt transgenic rat technology to additional inbred rat backgrounds, complete optimization of microinjection and transfer technologies.

It had been anticipated that investigators would desiree transgenic rats be created on inbred strains of rat. However, due to the inevitable delays that are encoutered as research proceeds, investigators had not come to the point where these experiments were desirable. Therefore, we turned out attention to another aspect of our proposal. Specifically, we initiated efforts to investigate the possibilities of generated germline mutations in endogenous rat genes, i.e. generating knockout rats.

The transgenic technique of pronuclear microinjection allows one to add a function to the cell as a result of expression of a gene linked to an active promoter. Due to the technique, this type of experiment is limited, therefore, to introducing gain of function mutations. The gain of function could be the introduction of dominant acting mutations of cellular genes some of which may have a dominant-negative activity. However, it is often the case that one needs to determine the effect of recessive mutations on the biology of an organ such as the mammary gland. In mouse transgenic technology, gene targeting approaches that allow one to generate mutations directly in cellular genes are frequently utilized. These gene targeting technologies rely on the availability of embryonic stem cells which, when reintroduced into the mouse embryo, can contribute to all lineages of the mouse including the germline. Mutations are generated within the DNA in the embryonic stem cell and then these cells are microinjected into blastocysts to generate chimeric mice. Chimeric mice are then bred to establish lines of mice carrying the desired mutation and these mice are then interbred to produce mice with mutations in both alleles of a given gene. In theory, this same technology could be applied to the rat thereby opening up the possibilities for generating recessive mutations in cellular genes of the rat directly. This would be most advantageous for deriving animal models for diseases because the genetic basis for many diseases is recessive mutations in particular cellular genes. There has been a long-standing effort by many investigators in the field, including Dr. Warren. to derive rat embryonic stem cells which retain the capacity to

contribute to the germline when reintroduced into rat blastocysts. Unfortunately, to date, despite these many effort, no one has derived such cells.

An alternative approach towards generating recessive mutations in the rat genome is suggested on the heels of the recent abilities to clone animals from somatic cells, as was the case for sheep named "Dolly" (32). In this approach one would generate the desired mutations by gene targeting in somatic cells that can be grown in culture. One cell clones carrying the desired mutations had been isolated and expanded, nuclei from these cells would be isolated and transferred into enucleated oocytes. The DNA from the somatic nucleus would then be deprogrammed in the environment of the oocyte and then, as the reconstituted embryo developed, be reprogrammed in keeping with developmental processes. Thus, a new individual is derived from the DNA of a somatic cell generating a identical copy, a clone.

During the past one and a half years, Dr. Warren in the laboratory has been investigating the feasibility of applying this technique to the rat, which we refer to as "rat cloning". The first requirement is that for the enucleation process, special epifluorescence equipment must be attached to the microinjection microscope. This capability allows one to visualize the metaphase plate in the oocyte which facilitates the complete removal of the pronucleus from the oocyte. Dr. Warren has purchased this equipment and become proficient at the enucleation step. The second requirement for this technique is the availability of suitable somatic cell lines in which to perform the gene targeting. Once targeted, these cells are the source of nuclei which are transferred into the enucleated oocyte. Although any somatic cell in theory can be used for this purpose, it is likely that cells of embryonic origin would be desirable because of the potential for more cell divisions in vitro before senescence. Embryonic fibroblasts are abundant, have good growth characteristics and are easy to establish in culture. Thus, Dr. Warren has established 6 lines of rat embryonic fibroblasts. The third requirement for this technique is that the nucleus from the somatic cell to be introduced into the enucleated oocyte. Two approaches are possible for this step, electrofusion or microinjection. The microinjection appears to be preferable because the embryo survival rate is 5 fold higher than the survival after electrofusion (33). Dr. Warren has purchased and set up the pieze-injector which is required for this microinjection manipulation and is currently becoming proficient at is use. The last requirement for this technique is that the recombinant oocytes be reintroduced into pseudopregnant recipients. However, prior to transfer, it is essential to culture the embryos to determine if they are viable. A question is how long the embryos can be cultured while maintaining their capacity to develop in utero. To this end, Dr. Warren has culture various stages of rat embryos and subsequently transferred them into the oviducts of pseudopregnant recipients to determine which combination of embryo stage and recipient stage is most supportive for embryonic development. Pregnancies were obtained from all stages of embryos used. This flexibility will permit the culturing of the recombined embryos through multiple cleavages before transfer. In sum, over the past year, Dr. Warren has set up many of the techniques that together are required for rat cloning.

During the past year, Dr. Warren participated in a workshop on rat cloning at Northwestern University where participants shared their experiences with various aspects of the technology. The workshop was hosted by Dr. Philip Innaccone. Work presented from the participating laboratories suggested that certain alterations in the originally described procedures of Wakayama might improve the chances for success. All participants decided to remain in close contact, believing that this "consortium effort" was more likely to result in success with rat cloning sooner that isolated efforts. Since that time, Dr. Warren has been incorporating the alterations into his procedure. Presently, he is focusing on developing the renucleation and activation steps. If he can

achieve cloning, then it will be possible to move on to target genes in the embryonic fibroblast, then transfer them to occytes from which can be derived rats with targeted genes.

6e. to Statement of Work Point E and F (Specific Aim 4): Develop and make available transgenic rat and breast cancer research communities specialized information databases.

The ability to communicate easily, effectively and efficiently with others in transgenic research and breast cancer research is essential in today's rapidly moving scientific world. To this end, many find it useful to communicate using the Internet where bulletin boards are reaching their highest popularity. In the first year of this grant, we joined two "clubs" on the Internet: Rodent Research and Embryo Mail. Over the ensuing years, we continued to be involved in dialogue through these electronic means and continue to find these services effective vehicles for rapid and informal discussion with our colleagues.

An extensive, up to date, easy to use directory of transgenic animal researchers and breast cancer researchers who use animal models as their primary model system is necessary to facilitate communication between these large groups of investigators Within the transgenic animal research community, there are several databases, such as the TBASE database operated by the Jackson Laboratories, which aim to list most knockout mice and transgenic animals in existence. Because of the existence of this first-rate database, we opted to encourage our users of transgenic rat services to supply information on their strains directly to Jackson Laboratories.

7. Key Research Accomplishments

- Evaluation of multiple variables on microinjection technique as it applies to rat embryos.
- Generation of a number of transgenic rat lines for breast cancer research.
- Establishment of a protocol for efficiently cryopreserving embryos from inbred and outbred rat strains.
- Establishment of a bank of frozen embryos from valuable transgnenic and congenic rat lines.
- Development of methods for rat cloning.

8. Reportable Outcomes

- Modified protocol for microinjection of rat embryos.
- Generation of multiple lines of transgenic rats carrying the following transgenes: Hras-Kras, Hras-Hras, MMTV-NeuN. MMTV-NeuT, MT-TGFα, MMTV-IGF2R.
- Protocol for cryopreserving embryos from superovulated rats.

- Establishment of a bank of embryos for the followingrat strains: Hras-Hras (2 lines), MMTV-NeuT, congenic rat strains 8370-line 122B, -line 35C, -line 173C, -line 56E, -line 94E.

- Manuscript:

  Helmuth, K.K. and A.E. Griep. A method for cryopreserving morulae from superovulated rats. In preparation.

- Abstracts:

  Helmuth, K.K. and **A.E. Griep**. 1996. Factors affecting viability of rat morulae during cryopreservation. Biol Reprod. 54 (Supplement 1): 185.

  Helmuth, K.K., J.R. Warren, J. Lohse, and **A.E. Griep**. Generation and cryopreservation of transgenic rat strains for breast cancer research. DOD Breast Cancer Research Program: An Era of Hope. Washington D.C., October 31-November 4, 1997.

9. Conclusions

During the years of this grant, we have made excellent progress in achieving our goals which are to generate transgenic rat models for breast cancer research, improve the efficiency and ease with which transgenic rats are produced, to develop effective methods for cryopreservation of rat embryos. We have generated multiple lines of transgenic rats that will be useful for breast cancer researchers including lines that ectopically express ras and neu oncogenes, the growth factor TGFα and the growth factor receptor IGF2R. All of these genes have been implicated in mediating the development of breast cancer in humans. These newly generated transgenic rats will provide researchers will a genetic model for the human disease with which investigators can assess the significance of the role of these genes in breast cancer. Furthermore, these strains will be important as animals for testing putative new therapies for the disease. We have developed an efficient protocol for the cryopreservation of rat mebyros from multiple strains of rat and established a bank of frozen embryos from some strains relevant to breast cancer research. This bank of embyros will be invaluable for "immortalizing" the strains for future use by investigators throughout the field. While transgenic rats have become a powerful tool for breast cancer research, it will be important to devise technologies to deliberately mutate endogenous genes. This is an essential technology to develop because the genetic basis of many diseases is recessive mutation in cellular genes and it is not possible to by dominant methods create the situation. Borrowing from the mouse, gene targeting in embryonic stem cells would seem to logical approach. However, rat embryonic stem cells that contribute to the germline when microinjected into the rat embryo have still not been isolated. Therefore, we have taken on the task of determining if rat cloning can be used as a means of generating rats carrying germline mutations in cellular genes. We have progressed to the point where each of the required manipulations is likely to be feasible in our hands; necessary reagents have been generated and required equipment obtained. Overall, our work has produced many valuable reagents for breast cancer research and heightened investigators awareness of the possibilities of using genetically manipulated rats in the research programs that are focused on understanding the underlying genetic basis for diseases like breast cancer and developing hew ways to combat the disease.

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10. Bibliography

(one hard copy of each is included as an attachment to this report)

Helmuth, K.K. and **A.E. Griep**. 1996. Factors affecting viability of rat morulae during cryopreservation. Biol Reprod. 54 (Supplement 1): 185.

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13.Appendix

Table 1: Comparison of transgenic rates of various constructs

Table 2: Comparison of transgenic rates in various injection media

Table 3. Live births from frozen-thawed embryos on inbred and outbred backgrounds

Table 4: Embryos frozen from various transgenic or congenic rat lines

Tab1e 1. Comparison of transgenic rates of various constructs

Construct	No. Embryos Transferred	No. Recipients	No. Live Offspring	No. Transgenics (%)
Hras - Kras	2686	65	61	5 (8.2%)
MMTV - NeuN	2858	82	79	7 (8.9%)
MMTV - NeuT	1224	33	16	1 (6.3%)
MT - TGFα	1539	52	68	2 (3.0%)
MMTV-IGF2R	1258	34	53	4 (6.6%)

Hras - Kras - transcriptional control regions of Hras fused to coding sequences of Kras gene

MMTV - NeuN - MMTV long terminal repeats fused to mutated neu proto-oncogene

MMTV - NeuT - MMTV long terminal repeats fused to wild type neu proto-oncogene

MT - TGFα - mouse metallothionein-I promoter fused to transforming growth factor alpha

MMTV-IGF2R- MMTV long terminal repears fused to insulin-like growth factor 2 receptor gene

Table 2. Comparison of transgenic rates in various injection media

Medium	No. Embryos Transferred	Recipients	No. Pregnant Recipients (%)	No. Live Offspring (%)	Transgenics (%)
M2	2319	115	80 (70%)	109 (4.7%)	13 (11.9%)
NG	759	24	19 (79%)	41 (5.4%)	7 (17.1%)
NP	714	16	9 (56%)	25 (3.5%)	1 (4%)
NPNG	586	17	13 (76%)	20 (2.9%)	1 (5%)
DPM2	750	12	9 (75%)	18 (2.4%)	1 (5.6%)
DPNG	229	5	5 (100%)	8 (3.5%)	1 (12.5%)
NG cyto-B	1067	35	23 (66%)	22 (2.1%)	1 (4.5%)

M2 - mouse M2
NG - mouse M2 lacking glucose
NP - mouse M2 lacking phosphate
NPNG - mouse M2 lacking glucose and phosphate
DM2 - mouse M2 containing glucosamine with reduced glucose
DPM2 - embryos presoaked in DM2 and then microinjected in M2
DPNG - embryos presoaked in DM2 and then microinjected in M2 lacking glucose
NG cyto-B - mouse M2 lacking glucose and containing cytochalasin B

Table 3. Live births from frozen-thawed embryos on inbred and outbred backgrounds

Morulae Transferred	No. Embryos Transferred	No. Recipients	No. Pregnant Recipients (%)	No. Offspring (%)*
Frozen-thawed Sprague Dawley SOM	231	14	13 (93%)	71 (32%)
Frozen-thawed Hras-Kras (on SD bkgd) SOM	106	7	7 (100%)	40 (38%)
Frozen-thawed Wistar Furth SOM	61	4	4 (100%)	20 (33%)
Frozen-thawed Copenhagen SOM	37	3	2 (67%)	5 (14%)

SOM=Morulae collected from superovulated females

*Percentages based on the number of embryos transferred into females that established pregnancy.

Table 4. Embryos frozen from various transgenic or congenic rat lines

Name of Line	Genetic Background	No. Embryos Frozen
Hras-Hras line 3648	Sprague-Dawley	317
Hras-Hras line 3160	Sprague-Dawley	324
MMTV-NeuT	Sprague-Dawley	272
8370 line 122B	Wistar-Furth	33
8370 line 35C	Wistar-Furth	17
8370 line 173C	Wistar-Furth	6
8370 line 56E	Wistar-Furth	60
8370 line 94E	Wistar-Furth	18

ATTACHMENTS

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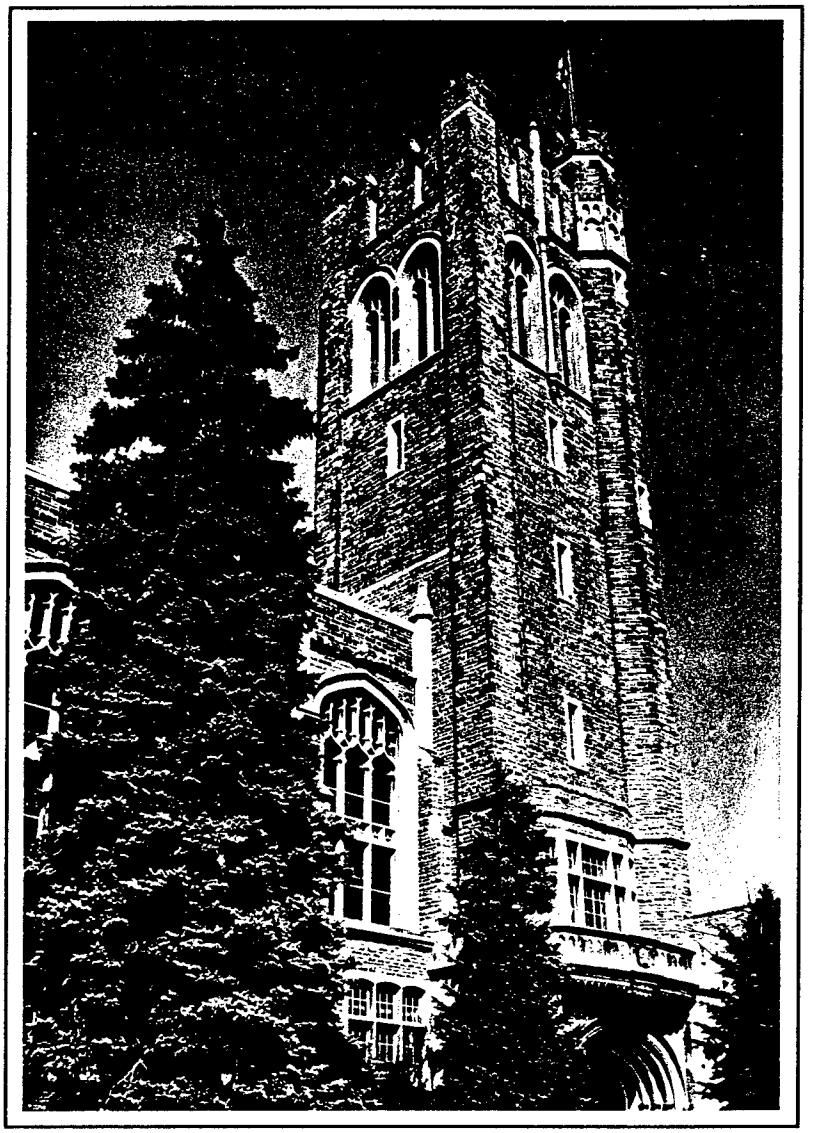

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513 SEASONAL EFFECTS ON POSTPARTUM REPRODUCTIVE ACTIVITY IN NATIVE CRIOLLO GOATS.

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The effects of season of kidding on postpartum intervals were evaluated in primiparous Criollo goats . The experiment was carried out at Rio Cuarto, Argentina (33° 08'S, 64° 20' W) using twelve females (two years of age) maintained under natural photoperiod and with a constant food supply to meet their metabolic requirements. All the dams suckled twins up to day 60 postpartum. Estrous behavior was determined twice a day by means of a teaser buck and blood samples were collected twice weekly to determine progesterone concentrations by radioinmunoassay. All of the fall kidding goats (n=6, delivered between April 11 to April 18) showed ovulation and estrus and postpartum intervals were 69.8 ± 5.5 and 73.5 ± 5.9 (mean ± SEM) days , respectively. Five out six first luteal phases were preceded by estrus and were considered of normal duration (progesterone concentration above 1 ng ml^{-1} for at least 10 days). These animals showed a second estrus (interestrous interval, 18.0 ± 2.0 days; mean ± SEM) and at least four of the subsequent luteal phases were of normal duration. None of the spring kidding dams (n=6, delivered between September 11 to September 23) showed ovulation or estrus for at least 120 days postpartum. These preliminary results would suggest the existence of overlapping effects of postpartum and seasonal anestrus in Criollo goats.

514 BIRTH OF LIVE MICE PRODUCED BY TRANSFER OF EMBRYOS DERIVED BY IN VITRO FERTILIZATION USING CRYOPRESERVED SPERMATOZOA.

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Production of mouse pups by in vitro fertilization (IVF) of oocytes with frozen-thawed spermatozoa has been reported. However, sperm survival has been either unspecified or low and published observations have been difficult to confirm. The objective of this study was to improve results using controlled-rate freezing. Epididymal sperm samples at concentrations of 2-4 x 10^7 sperm/ml and motility >60% were collected from B6D2F1 mice into phosphate-buffered saline (PBS) + 0.45% BSA. Sperm were diluted 1:2 with a solution of a cryoprotective additive (CPA) to yield a final concentration of 0.3 M raffinose + 0.2 M glycerol prepared either in PBS + 25% egg yolk (CPA-EY) or in PBS + BSA (CPA-BSA). Sperm samples (50 µl) in 0.25 ml plastic straws were cooled in a controlled-rate freezer from +22°C to 4°C at 5°C/min, held 2 min, then cooled at 20°C/min to -70°C, then plunged into liquid nitrogen. Frozen samples were thawed rapidly at >1,000°C/min, the cryoprotectant diluted rapidly, and the sperm incubated in PBS at 37°C for 30, 90 or 150 min. Survival at each interval was estimated from differential cell counts using a commercial fluorescent double dye (FertiLight™, Molecular Probes, Inc) Controls consisted of sperm in the initial diluent (unhandled) and sperm exposed to both types of CPA solution without freezing. Respective % viabilities of controls were: Unhandled: 43.7 ± 7.2, 34.2 ± 2.8 and 38.0 ± 4 8, CPA-EY: 40.9 ± 5.1, 31.8 ± 5.4 and 23.1 ± 4.9; CPA-BSA: 15.1 ± 0.5, 13.2 ± 3.4 and 10.1 ± 1.0. The corresponding % viabilities from 5 replicates after cryopreservation were: CPA-EY: 15.7 ± 1.4, 12.1 ± 0.9 and 11.3 ± 0.7; CPA-BSA: 5.7 ± 0.8, 5.1 ± 1.0 and 5.9 ± 1.8. Transfer of blastocysts produced by IVF with unhandled control (n=115 embryos), CPA-EY control (n=48) or cryopreserved sperm in CPA-EY (n=77) yielded live pups (50.4, 35.4 and 53.2%, respectively). These results represent a considerable improvement in the efficiency of mouse sperm cryopreservation.

515 FACTORS AFFECTING VIABILITY OF RAT MORULAE DURING CRYOPRESERVATION.

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The overall aim of this study was to develop an efficient procedure for cryopreservation of rat embryos. To address this aim, we evaluated the development of morulae from Sprague Dawley rats to blastocysts after exposure to 1)typical mouse embryo media or serum-free media, 2)serum-free media with varying osmolarities, 3)glycerol and sucrose, 4)conventional freezing procedures. Morulae cultured overnight in 100µl of HECM-1 (without the amino acids) supplemented with 7.5mM glucose developed to blastocysts at a higher rate (44%) than those cultured in M16 with 3.5mg/ml BSA (4.2%) or M16 with 1mg/ml polyvinylalcohol (0%). HECM-1 media with different osmolarities (283, 241, 227 mosmoles) were prepared by varying the NaCl concentration (98, 80, 73mM, respectfully). Development was greatest in HECM-1+7.5mM glucose+80mM NaCl (92% blastocysts). To determine the optimum cryoprotectant exposure time, morulae were dehydrated in 1.5M glycerol for either 15 or 20 min then rehydrated in either a 2-step procedure (5 min in 1.5M glycerol/0.3M sucrose then 5 min in 0.3M sucrose) or a 1-step procedure (10 min in 0.3M sucrose). No significant difference was examined between exposure times, however, fewer embryos developed to blastocysts when grouped 10-12 per drop as compared to those cultured 13 -18 per drop. The final objective was to examine viability after freezing and thawing. Morulae collected 90 h post fertilization were placed in 1.5M glycerol on ice for 15 min, loaded into 1/4cc straws and placed in a -4°C methanol bath. After 1-2 min, straws were seeded and held for 3-4 min. Embryos were frozen to -35°C at 0.3°C/min, held for 10 min then plunge into liquid nitrogen. Upon thawing, straws were exposed to room temperature for 2 min. Embryos were placed into a 1.5M glycerol/0.3M sucrose solution for 2 min, 0.3M sucrose for 5 min, then cultured in HECM-1+7.5mM glucose+80mM NaCl either with or without amino acids. There was no difference in development to blastocysts between either culture group (98% vs. 96%). It was determined that exposure of the straws to methanol for longer than 1-2 min before seeding was detrimental to the embryos. In vivo viability tests are now underway.

516 VIABILITY OF CRYOPRESERVED CAT PRE-ANTRAL FOLLICLES.

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About 3,000 pre-antral follicles can be recovered from a pair of domestic cat ovaries. *In vitro* maturation and fertilization of cat oocytes have met with some success, providing incentive for rescuing pre-antral oocytes. An ability to cryopreserve all follicular stages would allow more efficient use of valuable genetic material from rare felids that die abruptly or are ovariectomized for medical reasons. This study evaluated various cryoprotectants and compared fresh versus frozen-thawed viability of cat pre-antral follicles collected by mechanical dissection of ovaries. Follicles (40-90 µm dia.) were exposed to no cryoprotectant (control) or 1.5 M dimethylsulfoxide (DMSO), glycerol (GLYC), propanediol (PROH) or ethylene glycol (ETOH) for 15 min at 4°C before culturing in modified Eagle's Medium. Follicle viability was assessed immediately (0 h), at 18 h and 1 week using Hoechst 33458 and Trypan blue staining. Follicles with unstained oocytes and ≥90% unstained granulosa cells were classified as intact/viable. Percentages of intact follicles were similar ($P>0.05$) among control, DMSO, PROH and ETOH treatments at all time points, but were reduced ($P<0.05$) 7% after GLYC. DMSO and PROH were chosen as cryoprotectants for follicle cryopreservation. After cooling and addition of cryoprotectant, follicles were loaded into 0.25 ml straws and cooled at 0.5°C/min in an alcohol freezer to -7°C, seeded and further cooled at 0.5°C/min to -70°C before plunging into liquid nitrogen. After 24 h, straws were thawed, and viability was assessed at 0 h, 18 h and 1 week of culture by staining and measuring 5-bromo-2'-deoxyuridine (BrdU) uptake into oocytes. Of control (unfrozen) follicles, 31.4 ± 2.9%, 18.8 ± 1.9% and 16.2 ± 1.6% were intact, and BrdU uptake occurred in 22.1 ± 1.6%, 18.8 ± 2.0% and 23.6 ± 4.3%, respectively. Of follicles cryopreserved in DMSO, 17.2 ± 2.9%, 9.9 ± 1.5% and 9.1 ± 0.7% were viable, with BrdU uptake detected in 15.5 ± 5.4%, 11.4 ± 1.9% and 14.1 ± 2.8%, respectively. Of follicles cryopreserved in PROH, 14.9 ± 2.7%, 8.2 ± 1.4% and 8.1 ± 2.3% were viable, with BrdU uptake detected in 13.9 ± 4.5%, 11.9 ± 3.0% and 12.8 ± 3.7%, respectively. There were no differences in viability or BrdU uptake ($P>0.05$) between DMSO and PROH. Although viability was lower ($P<0.05$) for frozen-thawed than for fresh follicles, BrdU uptake was similar ($P>0.05$) among all groups. In summary, cat pre-antral follicles can withstand a standard oocyte cryopreservation protocol, retaining viability and metabolic activity in culture for up to 1 week after thawing.

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GENERATION AND CRYOPRESERVATION OF TRANSGENIC RAT STRAINS FOR BREAST CANCER RESEARCH

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Breast cancer is one of the leading cause of death among women, with one out of every nine women in the United States being predicted to develop this disease during her lifetime. Breast cancer is a disease in which numerous cellular and molecular genetic changes are thought to contribute to the multistaged progression of normal cells to a population of cells with unrestricted growth and metastatic potential. Deregulation of gene expression through chromosomal translocation or mutation in the regulatory elements of the gene, alterations in the activities of these gene products through mutation in the coding regions of the genes, or complete loss of these genes from the chromosome through mutation are considered to be mechanisms contributing to the failure of cells to maintain normal growth characteristics. The laboratory rat is an important model for studying breast cancer due to the many similarities in this disease between rats and humans. However, limited knowledge in manipulating the rat genome through transgenesis has prevented researchers from answering important questions in breast cancer research as well as in research on other human cancers. In the context of generating novel strains of rats for breast cancer studies, we proposed to carry out a series of detailed studies to optimize the many variables in transgenic manipulation, to extend transgenic rat technology to inbred rat strains, and to develop rat embryo cryopreservation.

Whereas a typical frequency of transgenesis in the mouse is 20%, the average frequency for transgenesis in the rat using mouse protocols was below 10%. Available evidence suggested that altering the medium composition by removal of glucose, replacement of glucose with glucosamine, removal of phosphate might enhance embryo viability and the frequency of transgenesis. Using one cell embryos from Sprague-Dawley rats for microinjection experiments, we evaluated how certain changes in medium composition affected embryo

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viability, as judged by the live birth rate after transfer into pseudopregnant recipients. Removal of glucose from the medium did not affect the lysis frequency upon microinjection but did significantly increase the percentage of pups born that were transgenic (17% versus 10%). Removal of phosphate from the injection and transfer medium resulted in a reduction in birth rate. Thus, while no glucose appears to be beneficial, no phosphate appears to be detrimental. Replacement of glucose with glucosamine was also detrimental for embryo development. We also evaluated the optimal numbers of embryos to be transferred per recipient and methods of entry into the oviduct. The optimal numbers of microinjected embryos transferred into Sprague-Dawley recipients was determined to be 40-50. Through these optimization studies, we have generated multiple lineages of rats transgenic for the neu oncogene, activated ras oncogene and TGFα, all of which have been implicated in breast cancer. Analyses of these rat models continues at present.

We have previously established routine procedures for the cryopreservation of valuable mouse strains. Adaptation of this technology for use with rat embryos will be critical for the long term success of transgenic rat programs. Using a newly reported rat embryo culture medium, which appears to provide optimal support for the development in vitro of early rat embryos, we have determined that glycerol is a suitable cryoprotectant. Furthermore, we have determined this medium supports the development of morulae from superovulated donor rats into blastocysts as efficiently as it does for morulae from naturally ovulated donor rats, being greater than 90% in each case. Morulae from either naturally ovulated or superovulated females can be frozen in glycerol from -4^{o}C to -35^{o}C at 0.3^{o}C/min then plunged into liquid nitrogen. Upon thawing and rehydration in sucrose containing medium, 86-91% of embryos are viable. Transfers of frozen/thawed morulae resulted from naturally ovulated females resulted in approximately 1/2 the numbers of live births as compared to transfers of unmanipulated embryos (46% versus 78%). Transfers of unmanipulated morulae from superovulated donors resulted in significantly lower frequency of liver births as compared to transfer of unmanipulated embryos from naturally ovulated donors (43% versus 78%). Transfers of cryopreserved morulae from superovulated donors resulted in similar live birth rates as compared to unmanipulated morulae from superovulated females (33% versus 43%). Thus, we have established a procedure for successfully cryopreserving rat morulae using superovulated donors. While the frequency of live births from superovulated morulae is significantly less than that from naturally ovulated donors, the use of superovulated donors reduces significantly the size of the rat colony necessary to support cryopreservation program and the time required to generate and isolate embryos for cryopreservation.

Therefore, through our studies, we have developed procedures to enhance the ability to generate transgenic rats and the means of long term preservation of these valuable strains of rats. In the process, we have generated numerous new strains of transgenic rats which are being studied actively in breast cancer laboratories. Efforts are underway to adapt these procedures to inbred strains of rats used in breast cancer research. The results of these studies not only improve the capacity to use the rat as a model system for studying the molecular aspects of breast cancer but also for studying other cancers where rat is the species of choice.

A Method for Cryopreserving Morulae From Superovulated Rats[1]

5 *Cryopreservation of rat morulae*

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15

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ABSTRACT

The aim of this study was to develop an efficient procedure for freezing rat embryos from superovulated donors using medium recently recommended for rat embryos (R1ECM) and conventional cryopreservation procedures. Blastocyst development of unmanipulated morulae from naturally ovulated Sprague Dawley females (NOM) and superovulated females (SOM) in R1ECM was not significantly different (100% vs. 95.6%). In vitro and in vivo developmental capabilities of NOM and SOM were evaluated after freezing and thawing. Morulae were frozen in glycerol from -4°C to -35°C at 0.3°C/min then plunged into liquid nitrogen. Upon thawing and rehydration, 85.8% NOM were viable as compared to 91.1% SOM. Addition of amino acids to R1ECM significantly improved development of thawed morulae to blastocysts (82.5% vs. 73.8%). Transfers of frozen/thawed NOM into day 3 pseudopregnant females yielded a significantly lower number of live births as compared to births from transfers of unmanipulated NOM (46.2% vs. 77.8%). Transfers of frozen/thawed SOM yielded similar live births in comparison to births from transfers of unmanipulated SOM (33.9% vs. 42.9%). Thus, this is the first report of an efficient, successful cryopreservation protocol for rat embryos using superovulated donor females.

INTRODUCTION

Cryopreservation is accepted as a successful means for long-term storage of highly valued embryos. Historically, mice have been the model of choice for investigations using small laboratory animals. The transgenic and knockout technology widely used in the last two decades has resulted in a large increase in the number of new mutant strains that need to be preserved. A bank of frozen embryos from transgenic animals provides insurance against their loss due to breeding failure or disease and also eliminates high maintenance and breeding costs. Recently, there has been a rise in the use of transgenic rats as laboratory models. Therefore it is likely that the ability to preserve rat embryos from transgenic lines in an efficient and cost effective manner will be desirable by investigators in the very near future.

In the 1970's, successful cryopreservation of embryos has produced live young in many species including cattle [1], sheep [2], rabbits [3], goats [4] and mice [5, 6]. Since these studies, many investigators have examined factors involved in the cryopreservation protocols such as type and concentration of cryoprotectant, freezing and thawing temperatures and rates. Through these extensive investigations, routine cryopreservation techniques have been developed for each species. For rat embryo cryopreservation, few studies have been undertaken. Whittingham [7] reported that 8-cell CFHB random-bred rat embryos can survive conventional freeze-thaw procedures (65-73% survival) with fair in vitro development (50% blastocysts). Miyamoto and Ishibashi [8] reported slightly lower levels of in vitro development for 8-cell embryos from Wistar rats when frozen in various glycols (21-52% blastocysts). Kasai et al. [9] froze morulae from Wistar rats in various cryoprotectants, including glycerol, and saw modest in vitro development (65-71% blastocysts) as well as moderate

in vivo development (33-50%); however, the number of embryo transfers performed was small. Utsumi et al. [10] obtained 83% survival after freezing Wistar morulae and early blastocysts in 1M adonitol. Additionally, they reported 42.2% recovery as live offspring.

In these few publications, investigators used non-conventional tools for freezing, making their techniques difficult to reproduce in typical laboratories. Embryos were frozen in either glass test tubes or in ampules and only one report used conventional, plastic 0.25 ml straws. The cooling method applied in some of these studies was performed by adding solid CO_2 to an ethanol bath. Most laboratories freezing embryos currently use a controlled rate programmable freezer which supplies both consistent and accurate cooling rates. Finally, in all these previously mentioned studies the investigators collected embryos from naturally ovulated donor females, which requires a large breeding stock and therefore, higher animal maintenance costs and additional labor on the part of the investigator to produce and isolate embryos for cryopreservation.

It is well known that medium composition plays an important role in embryonic development. In procedures used previously for rat embryo cryopreservation, embryos were manipulated in various media such as modified Dulbecco's PBS, modified Kreb's-Ringer solution and Ham's F10. Since these procedures were published, newer studies have reported culture conditions for rat embryos that significantly improved developmental capabilities in vitro. These studies were based on the observation that hamster embryos overcome the 2-cell block when cultured in medium lacking phosphate and glucose [11]. Using this hamster embryo culture medium

(HECM-1), Kishi et al. [12] were the first to report that 1-cell rat embryos can be cultured past the 2-cell stage and achieved 20% blastocysts development. Miyoshi et al. [13] investigated the suitability of this medium further and found that supplementation with glucose and deletion of the amino acids supports 67% blastocyst development from 1-cell rat embryos. In subsequent experiments, Miyoshi et al. [14] reported that the addition of MEM amino acids to this culture medium, called R1ECM, supported 90% blastocyst development from cultured 1-cell embryos.

Because existing protocols for rat embryo cryopreservation used atypical tools and less than optimal culture medium, we asked if R1ECM would support high percentages of blastocyst development of rat morulae frozen by procedures commonly used for mouse and other species. Initially, we confirmed that Sprague Dawley morulae developed to blastocysts in R1ECM and then we used a HEPES-buffered variation of this medium to prepare a solution for cryopreservation. Using these media, we evaluated in vitro and in vivo development of rat embryos after cryopreservation in plastic straws using a controlled rate freezer. Lastly, we assessed the suitability of superovulated rats as donors of morulae for this cryopreservation procedure.

MATERIALS AND METHODS

Medium

Two types of media are described, collection medium and culture medium. The collection medium was used to flush embryos from the oviduct and uterus. This collection medium is a minor modification of the rat embryo culture medium R1ECM [14]. In our experiments, the collection medium has 80mM NaCl (increased from 78.8mM in standard R1ECM) and was adjusted for use in air by

decreasing the sodium bicarbonate concentration to 2mM and adding 10mM HEPES. This collection medium, designated R1ECM-HEPES, was prepared without sodium pyruvate and frozen in 10 ml aliquots. On the day of the experiment, an aliquot was thawed at room temperature and 0.5 mM sodium pyruvate added.

A culture medium (R1ECM) designed for rat embryos was prepared according to details in Miyoshi et al. [14] except NaCl concentration was increased from 78mM to 80mM. Minimal essential amino acids were added in Experiment 3, to reproduce the culture medium used by Miyoshi et al. [14]. The culture medium was prepared fresh daily and filtered sterilized. For embryo culture experiments, 100µl drops of R1ECM were covered with light white mineral oil (Sigma Chemical Company, St. Louis, MO) and equilibrated 1-3 h at 37°C in 5% CO_2:95% air prior to embryo placement. Ten to fifteen embryos were cultured per 100µl drop. Initially, development was scored at 24, 42, and 48 h. The percentage of morulae that had progressed to blastocysts was greatest at the 42 h timepoint (ie. some blastocysts had collapsed by 48 h in culture). Hence, in all subsequent experiments, data were collected at 42 h.

Animals

Sprague Dawley outbred rats were purchased from Harlan Sprague Dawley (Madison, WI) and used to establish a breeding colony. Rats were maintained in 14 h light:10 h dark cycle. All animal handling and experimental procedures were approved by University of Wisconsin Animal Care and Use Committee.

Production/Collection of embryos

Embryos were produced by either natural ovulation or superovulation. For natural matings, vaginal smears were done on sexually mature females. Those exhibiting signs of estrus were mated to males. The following morning, females were checked for the presence of a vaginal plug or sperm (plug date=day 1). As per VetrePharm's instructions for superovulation, osmotic minipumps (Alza, Palo Alto, CA) filled with 0.2 ml FSH (NIH-FSH-P1:VetrePharm, London, Ontario) were inserted s.c. into 28-32 day old females between 9-11 am on Day -2. On day 0, 0.2 ml LH (VetrePharm, London, Ontario) was injected i.p. and females were mated to males. The following morning, females were checked for copulatory plugs and the minipumps removed. Morulae (8 to 16-cells) from either superovulated females (SOM) or naturally ovulated females (NOM) were collected in R1ECM-HEPES by flushing the oviducts and uteri on the afternoon of day 4, approximately 86-92 h post-fertilization (assuming fertilization takes place near the midpoint of the dark cycle).

Freezing

Freezing and thawing procedures were a modification from Kasai et al. [9]. Morulae were placed in a solution of 1.5M glycerol (prepared in R1ECM-HEPES) on ice for 15 min. During this time period, 0.25 ml straws were loaded in a similar procedure as described by Leibo et al. [15] except no diluent was loaded into the straws. Briefly, each straw was loaded in the following order: 12 mm of glycerol followed by a 10 mm air bubble then 10 mm of glycerol containing the embryos (10-40 morulae per straw) followed by another 10 mm air bubble then the remainder of the straw was filled with glycerol. Straws were returned to ice after loading. After 15 min, straws were completely submerged

in a -4°C methanol bath in a BioCool III freezer (FTS Systems, Stone Ridge, NY). During a 5 min equilibration period, straws were seeded in two places, on the solutions above and below the glycerol drop that contains the embryos. These seedings took place between 1-3 min after initial exposure to methanol. Straws were then frozen from -4°C to -35°C at a rate of 0.3°C/min. Straws were held at -35°C for 10 min, plunged into a dewar flask of liquid nitrogen then transferred into a liquid nitrogen tank for storage. Embryos were stored for 24 h - 9 wks.

Thawing and Removal of Cryoprotectant

For thawing, straws were removed from liquid nitrogen and placed horizontally on a test tube rack at room temperature for 2 min. Embryos were expelled from the straws and glycerol was removed in a two-step process. Embryos were exposed to 2 min of 1.5M glycerol/0.3M sucrose (prepared in R1ECM-HEPES) then 5 min of 0.3M sucrose (prepared in R1ECM-HEPES). Embryos were rinsed in R1ECM either with or without amino acids (MEM Amino Acids Solution (50X), without L-glutamine, Gibco, Grand Island, NY). Lyzed and degenerate embryos were discarded and viable embryos cultured. Development was scored at 42 h. For in vivo experiments, embryos were cultured for 1-3 h and viable embryos transferred into pseudopregnant recipients.

Embryo Transfers

To obtain pseudopregnant recipients, vaginal smears were taken from Sprague Dawley females (at least 3 months of age). Females in estrus were placed with vasectomized males and the following morning examined for the presence of a vaginal copulatory plug (plug date=day 1). On Day 3, recipients were

anesthetized with Avertin [16, 17] at a dose of 0.016ml/g body weight. Two-sided embryo transfers were performed similarly as published for the mouse [18]. Briefly, the ovary, oviduct and tip of uterine horn were exposed through a dorsal incision in the skin and body wall. Using a 22 gauge needle, a hole was made in the uterus. The embryos were picked up with a capillary pipette and inserted into the uterus through this hole. The reproductive tract was then returned to the body cavity. The body cavity was closed with suture and the incision closed with wound clips. Recipients were allowed to deliver and the number of pups born was recorded.

Experimental Design

Experiment 1 examined the effects of osmolality on blastocyst development from NOM. Based on experiments by Miyoshi et al. [13], morulae were cultured in R1ECM that was adjusted to 227 (73mM NaCl), 241 (80mM NaCl) or 283 (98mM NaCl) mosmol by altering the NaCl concentration. Osmolality of each solution was verified by measuring the freezing point using an osmometer. Embryos from each donor were equally distributed across each treatment to minimize an individual donor effect. Initially, development was scored at 24, 42, and 48 h. The percentage of morulae that had progressed to blastocysts was greatest at the 42 h timepoint (ie. some blastocysts had collapsed by 48 h in culture). Hence, in all subsequent experiments, data were collected at 42 h.

Experiment 2 determined the viability of NOM after exposure to freezing and thawing solutions without changing the ambient temperature. Glycerol was selected as the cryoprotectant based on previous success in the rat [9]. Morulae were dehydrated in 1.5M glycerol for 15 min or 20 min then the cryoprotectant was removed in either a two-step process (1.5M glycerol/0.3M

sucrose for 2 min then 0.3M sucrose for 5 min) or a one-step process (0.3M sucrose for 10 min). To determine if dehydration at a lower temperature than room temperature effects embryo viability, morulae were dehydrated in 1.5M glycerol on ice then rehydrated in a two-step process. Untreated morulae were also collected and cultured to serve as controls. Embryos from each donor were distributed across at least two treatments to minimize an individual donor effect. Embryos were cultured in R1ECM for 42 h. Development was assessed by counting the number of blastocysts that had formed and then calculating the percentage of morulae that had developed into blastocysts.

Experiment 3 examined in vitro viability of morulae after freezing and thawing. Viable frozen/thawed NOM were cultured in R1ECM either with or without minimal essential amino acids. Blastocyst development was assessed as in Experiment 2.

Experiment 4 assessed in vitro development of unmanipulated NOM and SOM. Embryos were collected and cultured in R1ECM. Blastocyst development was assessed as in Experiment 2.

Experiment 5 assessed capacity of cryopreserved morulae to develop to term after embryo transfer. Viable frozen/thawed NOM and SOM were transferred into day 3 pseudopregnant recipients and the number of offspring born from each were compared. Unmanipulated control NOM and SOM were transferred immediately into the uteri of recipients to establish baseline in vivo survival rates.

Statistical Analysis

Statistical analysis was performed on the percentage of embryos that developed to blastocysts in Experiments 1 and 2 by analysis of variance. Percentages of blastocysts developing in Experiments 3 and 4 were analyzed by paired t-test. Comparison of pregnancy rates was performed by Fischer's Exact Test. Chi-square test was performed on the percentages of offspring born from the transfers of frozen/thawed embryos in comparison to unmanipulated control transfers into day 3 pseudopregnant recipients.

RESULTS

Effect of osmotic pressure on development of rat morulae from naturally ovulated donors

Table 1 shows the effects of osmotic pressure on blastocyst development in R1ECM with varying NaCl concentrations. Although there was a numerically higher developmental rate at 241 mosmol (equivalent 80mM NaCl), there were no significant differences among the developmental rates in the three treatments. In subsequent experiments, R1ECM@80mM NaCl was used as the culture medium.

Effect of cryoprotectant, temperature and dehydration/rehydration regimen on rat morulae development

As shown in Figure 1, exposure of rat morulae to 1.5M glycerol and 0.3M sucrose did not compromise viability. Morulae were dehydrated in glycerol for either 15 or 20 min, then rehydrated in either a one-step or a two-step process. No significant differences were found in the rates of development when treatments were compared to each other or when compared to untreated controls.

In these previous experiments, exposures of morulae to solutions used for dehydration/rehydration regimens were performed at room temperature. To determine if dehydration of the embryos at a lower temperature would adversely affect their capability to subsequently develop in culture, embryos were exposed to freezing medium at 0°C. In this experiment, morulae were dehydrated in 1.5M glycerol at 0°C and rehydration in a two-step process (1.5M glycerol/0.3M sucrose for 2 min then 0.3M sucrose for 5 min) at room temperature. As shown in Figure 1, development to blastocysts after exposure to glycerol at 0°C was not significantly different from other treatments or untreated controls. For subsequent freezing experiments, embryos were dehydrated for 15 min in glycerol that was cooled on ice and rehydrated in a two-step procedure after thawing.

Survival and developmental rates of rat morulae from naturally ovulated donors after cryopreservation

Viability of cryopreserved morulae from naturally ovulated donor females was determined after thawing. Upon thawing 10 different straws, 145 morulae were recovered. Of these, 128 had normal morphological 8 - 16-cell appearance and were classified as viable (88.3% survival; Table 2). Based on the enhanced developmental rate of rat embryos cultured in R1ECM with minimal essential amino acids [14], viable morulae within a given straw were cultured in R1ECM either with or without minimal essential amino acids. In culture medium containing amino acids, there was significantly greater blastocyst development of frozen/thawed NOM (52 of 63 embryos; 82.5%) as compared to frozen/thawed NOM cultured in medium without amino acids (48 of 65 embryos; 73.8%).

In vitro development of rat morulae from naturally ovulated and superovulated donors

The use of FSH to stimulate a superovulatory response resulted in an average of 45 intact morulae per donor (Table 3). This is a three fold greater yield as compared to the average yield of 14 morulae from naturally ovulated donors for this experiment. In vitro blastocyst development from unmanipulated NOM and SOM is also shown in Table 3. Morulae produced by superovulation develop to blastocysts as well as morulae produced by naturally ovulated donors (95.6% vs. 100%; $p>0.05$).

In vivo survival rates of cryopreserved embryos

Determination of the optimal stage of pseudopregnancy for uterine transfers of morulae was examined in experiment 5. Synchronous transfers of unmanipulated NOM into day 4 pseudopregnant recipients resulted in 66.7% pregnancy rate with 11 pups born out of 28 embryos transferred into pregnant females (39.3% live young; Table 4). Asynchronous transfers of unmanipulated NOM into day 3 recipients yielded 100% pregnancy with 42 pups from 54 transferred embryos (77.8% live young). Given this sigificant difference, asynchronous day 3 pseudopregnant females were used as recipients for frozen/thawed morulae.

Table 4 also compares developmental capabilities of frozen/thawed SOM and NOM. Morulae from superovulated donors survived the freeze/thaw process as well as NOM (91.1% vs. 85.8%, respectfully). When frozen/thawed NOM were transferred into day 3 pseudopregnant recipients, 86.7% became pregnant. Out of the recipients in which pregnancy was established, 46.2% of the transferred morulae developed into live pups. This in vivo developmental rate

is significantly lower than from offspring produced by transfers of unmanipulated NOM (46.2% vs. 77.8%; $p<0.05$). When frozen/thawed SOM were transferred into day 3 pseudopregnant recipients, 95.2% became pregnant. The developmental rate of pups produced from transferred frozen/thawed SOM (111 out of 327; 33.9%) was not significantly different from that of control transfers of unmanipulated SOM (30 out of 70; 42.9%).

DISCUSSION

In this study, we re-investigated existing procedures for rat embryo cryopreservation. Our goals were to establish a successful procedure that is both time and cost effective as well as compatible with equipment most commonly used for embryo cryopreservation in other species. Through investigations of media, freezing and thawing procedures, ovulation and transfer protocols, we have designed a procedure using morulae from superovulated outbred Sprague Dawley donors that results in successful recovery of 33.9% live pups after embryo cryopreservation. Our results on embryonic survival, in vitro and in vivo development after cryopreservation by conventional freezing methods are at least comparable to and in many cases exceed previous reports. In summary this procedure is: prepubertal Sprague Dawley rats are superovulated with continuous infusion of purified FSH. Rat morulae (age 86-92 h post fertilization) are collected, placed in glycerol (prepared in R1ECM-HEPES), frozen in plastic straws from -4°C to -35°C at 0.3°C/min then stored in liquid nitrogen. Morulae are thawed, rehydrated in a two-step sucrose procedure, cultured for 1-3 h in R1ECM+amino acids then transferred into day 3 pseudopregnant recipients.

It is well known that various medium components can have a negative impact on in vitro development of certain rodent embryos including hamster [11, 19] and rat [12, 13]. Specifically in the rat, Kishi et al. [12] were the first to report that 1-cell rat embryos must be cultured in the absence of phosphate and glucose to develop past the 2-cell stage. Using this same medium, Miyoshi et al. [13] found an osmolality of 244 mosmol (equivalent to 80.05mM NaCl) to be optimum for blastocyst development from 1-cell embryos in culture. However, it was not clear whether small changes in osmolality would affect the morulae to blastocyst transition. Our data show that varying the NaCl concentration from 80mM had no effect on blastocyst development from cultured morulae. Therefore, embryos in the morula to blastocyst transition do not appear to be as sensitive to changes in osmolality as earlier stage embryos. Furthermore, Miyoshi et al. [13, 14] found that medium supplemented with glucose, defined as R1ECM, supported high blastocyst development from 1-cell rat embryos in the absence and presence of amino acids (67% - 90%). This was an improvement from previous data in which only 10% blastocyst development was achieved using the same medium without glucose [12]. These authors did not specify at which developmental stage glucose is required. However, in our initial experiments, we found R1ECM without glucose unable to support blastocyst formation from cultured morulae (data not shown). In summary, our investigation of culture media indicates that R1ECM provides good culture conditions for morulae from Sprague Dawley rats which is consistent with results from in vitro development of embryos on the Wistar background [13, 14].

Cryopreservation involves many steps including dehydration of embryos in a cryoprotectant, actual freezing to sub-zero temperatures and dilution of

cryoprotectant in a rehydration solution upon thawing. We chose glycerol as the cryoprotectant and sucrose to remove the cryoprotectant based upon reported success in previous rat embryo cryopreservation experiments [9, 20]. Other cryoprotectants such as ethylene glycol and DMSO have been investigated for use in slow freezing rat embryos without enhanced success [7-9]. In our hands, glycerol and sucrose proved to be suitable for rat morulae because neither one individually or collectively negatively affected developmental capabilities as compared to untreated controls. Many cryopreservation procedures in various species include chilling the cryoprotectant prior to embryo placement. Therefore, a treatment group in which morulae were exposed to glycerol at 0°C was included in this experiment. Development of these morulae to blastocysts was not significantly different in comparison to treatments performed at room temperature. Additionally, we found no differences in development after exposure of morulae to either a one-step or two-step rehydration procedure. All of these experimental variables yielded only insignificant differences on developmental capabilities of rat morulae. This may in part be due to the use of R1ECM as the medium to prepare the glycerol and sucrose solutions.

In the cryopreservation procedure reported by Kasai et al. [9], rat morulae were frozen from -4°C to -50°C at 0.33/min then to -75°C at 1°C/min. This procedure requires approximately 3 h of slow freezing time and a freezer that can achieve -75°C. Stein et al. [20] attempted to freeze rat blastocysts to -30°C only but were unsuccessful in obtaining viable embryos upon thawing. Because many common controlled rate freezers only have the capacity to freeze to -40°C and freezing procedures in other species typically freeze to -35°C before plunging into liquid nitrogen, it was also important for us to

determine if it is possible to successfully cryopreserve rat embryos using a higher freezing endpoint before plunging into liquid nitrogen. Upon reinvestigation we found that morulae isolated from naturally ovulated donors can survive freezing from -4°C to -35°C at 0.3°C/min before plunging into liquid nitrogen. This procedure yielded 85.8% - 88.3% viable embryos after thawing. In vitro development of cryopreserved morulae in the presence of amino acids resulted in a significant increase in blastocyst formation as compared to development in medium without amino acids (82.5% vs. 73.8%). These results confirm previous reports that amino acids may be beneficial to cultured rat embryos [14, 21]. Based on our success at freezing rat morulae to -35°C before plunging into liquid nitrogen, slow freezing to -75°C is not necessary under conditions we used. This success may be due to the medium composition of R1ECM, the use of straws rather than glass ampules or a combination of these two factors. Thus, rat morulae can be frozen and recovered successfully using a freezing regimen that is compatible with commonly used controlled rate freezers and saves 1 hr. of time as compared to the procedure by Kasai et al. [9].

In previous reports on rat embryo cryopreservation, investigators froze embryos generated by natural ovulation [7-10, 20]. Use of natural ovulation procedures requires one to maintain a large colony of donor females and stud males to produce enough embryos for cryopreservation. Use of natural ovulation procedures also requires a large time commitment on the part of the investigator to set up and score matings, and to collect embryos from large numbers of donors. Superovulation maximizes the animal's reproductive capabilities and minimizes personnel's efforts required to maintain the animal

colony and produce embryos suitable for freezing. Therefore, it would be advantageous to use morulae from superovulated donors for cryopreservation. To date, no one has determined if morulae from superovulated females were of sufficient numbers and quality to use for cryopreservation. Two basic methods for ovarian stimulation can be used with rats; Pregnant mare's serum gonadotropin and FSH. Pregnant mare's serum gonadotropin (PMSG) has been used to stimulate a superovulatory response in immature Sprague Dawley females, however, ovulation rates were modest (31±10 oocytes; [22]). Alternately, use of a continuous infusion of FSH induces a stronger, less variable response in the same studies (76±12 oocytes). Armstrong and Opavsky [22] further report that recovery of advanced preimplantation embryos from FSH induced rats yields 52±8 morulae and blastocysts on day 5. In our hands, continuous infusion of FSH stimulated a superovulatory response in immature Sprague Dawley females yielding 42±3 morulae on average per donor. This superovulation regimen produced an almost 4 fold increase in morulae production in comparison to natural ovulation which produced 11±1 morulae per donor throughout our studies.

Despite the high number of morulae produced by FSH stimulation, the long-term quality and developmental capability of these embryos may be an issue. Armstrong and Opavsky [22] reported that oocytes generated by PMSG had a significantly higher proportion of degeneration on day 1 as compared to oocytes generated by FSH (56% vs. 0%). In our studies using later stage preimplantation embryos, blastocyst development from NOM and SOM were similar after 42 h in culture indicating no detrimental effects of FSH-induced ovulation on short-term in vitro development. Additionally, we found no difference in survival rates of NOM and SOM after cryopreservation (85.8% for

NOM, 91.1% for SOM). However, we did observe that transfers of unmanipulated NOM and SOM into pseudopregnant females resulted in a significantly lower percentage of live births (77.8% vs 42.9%). This developmental difference implies that SOM lack the quality necessary to attain the full developmental potential that NOM exhibit. Although hormones for superovulation are costly and there is less than 2 fold decrease in in vivo development, both factors are clearly offset by the 3 fold or more increase in yield per donor and substantial savings in animal maintenance costs and personnel time.

Finally, previous investigators have examined in vivo development of cryopreserved rat embryos. In reports using slow freezing procedures, in vivo survival percentages varied greatly from 9% to 50%, and the number of embryos transferred was low [7, 9, 20]. Vitrified rat embryos have also been transferred to obtain live offspring, achieving 40% development [10, 23]. Details on establishment of pseudopregnancy and embryo transfers were not included in these studies. And, more importantly, no investigations of in vivo developmental capabilities of cryopreserved SOM have been reported. First, we have shown that day 3 pseudopregnant females established by mating to vasectomized males allowed for a higher percentage of births from transferred unmanipulated NOM as compared to day 4 pseudopregnant females (77.8% vs. 39.3%). Second, we determined that a significantly lower in vivo survival rate is achieved by transferring frozen/thawed NOM into day 3 pseudopregnant recipients as compared to control NOM (46.2% vs. 77.8%). This indicates that cryopreservation has a prolonged effect on NOM that is evident upon transferring into recipients. Last, we have shown that in vivo survival of frozen/thawed SOM is comparable to control SOM (33.9% vs. 42.9%)

indicating the loss of embryonic survival is due to the ovulation regimen not the cryopreservation procedures. Therefore given the fact that the yield of viable morulae from superovulated donors is almost 4 times that from naturally ovulated donors and in vivo survival rates of frozen/thawed SOM are only 2 fold less than NOM, it is more efficient to produce rat morulae by superovulation for cryopreservation procedures.

In conclusion, we verified that R1ECM provides good culture conditions for rat morulae from Sprague Dawley females. Morulae obtained from naturally ovulated and superovulated donors can be cryopreserved in glycerol in plastic straws from -4°C to -35°C before plunging into liquid nitrogen. After thawing, 73.8 - 82.5% frozen morulae develop into blastocysts. Therefore, morulae from superovulated donors survive cryopreservation as well as naturally ovulated morulae and develop in vitro comparably. The ultimate goal of any cryopreservation procedure, high percentage of live offspring, can be achieved when NOM and SOM from outbred Sprague Dawley rats are cryopreserved. This procedure described herein achieves this goal. We are now extending our studies to determine if morulae from various inbred strains can be successfully cryopreserved using FSH-induced ovulation and these cryopreservation procedures.

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FIGURE LEGENDS

Figure 1. Morulae were dehydrated in glycerol for either 15 or 20 min at room temperature (G15 or G 20) or for 15 min on ice (G15 0°C). Embryos were then rehydrated in either a two-step process: 1.5M glycerol/0.3M sucrose for 2 min then 0.3M sucrose for 5 min (GS 2 min, S 5 min); or in a one-step process: 0.3M sucrose for 10 min (S 10min). Ten to fifteen morulae were cultured in 100µl drops of R1ECM at 37°C in 5% CO_2 and development to blastocysts recorded after 42 h. Untreated morulae were also cultured to serve as controls. Each bar represents the mean ± SEM of data from at least 6 replicates. No significant differences were found between untreated controls and treatment groups ($p>0.05$).

Table 1. Effect of osmotic pressure on development of rat morulae to blastocysts in vitro.

Culture Medium	Osmolality (mosmol)	No. Embryos Cultured	No. Blastocysts at 42 Hours (%)
R1ECM @ 98 mM NaCl+	283	50	43 (86.0)[a]
R1ECM @ 80 mM NaCl+	241	50	46 (92.0)[a]
R1ECM @ 73 mM NaCl++	227	45	38 (84.4)[a]

+Data from 5 replicates

++Data from 4 replicates

[a]not significantly different ($p>0.05$)

Table 2. Survival and developmental rates of rat morulae from naturally ovulated donors after cryopreservation.

No. Morulae Frozen*	No. Morulae Recovered after Thawing	No. Viable Morulae (%)**	Culture Medium	No. Frozen/thawed Morulae Cultured	No. Blastocysts at 42 hours (%)
151	145	128 (88.3)	R1ECM	65	48 (73.8)[a]
			R1ECM + amino acids	63	52 (82.5)[b]

*Morulae were collected from 15 naturally ovulated females. Embryos were pooled and frozen.

**Morulae were recovered from 10 different straws. Viablity was assessed after thawing and rehydration. Lyzed and degenerate embryos were discarded. Percentage viable was calculated from the number of morulae frozen. Viable embryos within a given straw were divided between the two culture groups.

[a,b]Values with different superscripts within each column are significantly different ($p<0.05$)

Table 3. In vitro development of rat morulae produced by natural ovulation and superovulation

Ovulation Regimen	No. Embryos Cultured	No. Blastocysts at 42 hours (%)
NOM+	42	42 (100)[a]
SOM++	90	86 (95.6)[a]

+NOM = morulae collected from 3 naturally ovulated females.

++SOM = morulae collected from 2 superovulated females.

[a]not significantly different ($p>0.05$)

Table 4. Development of frozen/thawed morulae after transfer into pseudopregnant females.

	No. Morulae Frozen	No. Morulae Recovered After Thawing	No. Viable Morulae Transferred(%)*	Fraction of Recipients Pregnant (%)	No. Offspring Born (%)**
Transfer of unmanipulated NOM into day 4 recipients	NA	NA	43	2/3 (66.7)	11 (39.3)
Transfer of unmanipulated NOM into day 3 recipients	NA	NA	54	4/4 (100)^{a}	42 (77.8)^{a}
Transfer of frozen/thawed NOM into day 3 recipients	254+	244	218 (85.8%)	13/15 (86.7)^{a}	84 (46.2)^{b}
Transfer of unmanipulated SOM into day 3 recipients	NA	NA	70	5/5 (100)^{a}	30 (42.9)^{b,c}
Transfer of frozen/thawed SOM into day 3 recipients	370++	367	337 (91.1%)	20/21 (95.2)^{a}	111 (33.9)^{c}

NOM = morulae collected from naturally ovulated females; SOM = morulae collected from superovulated females

+Morulae collected from 18 naturally ovulated donors.

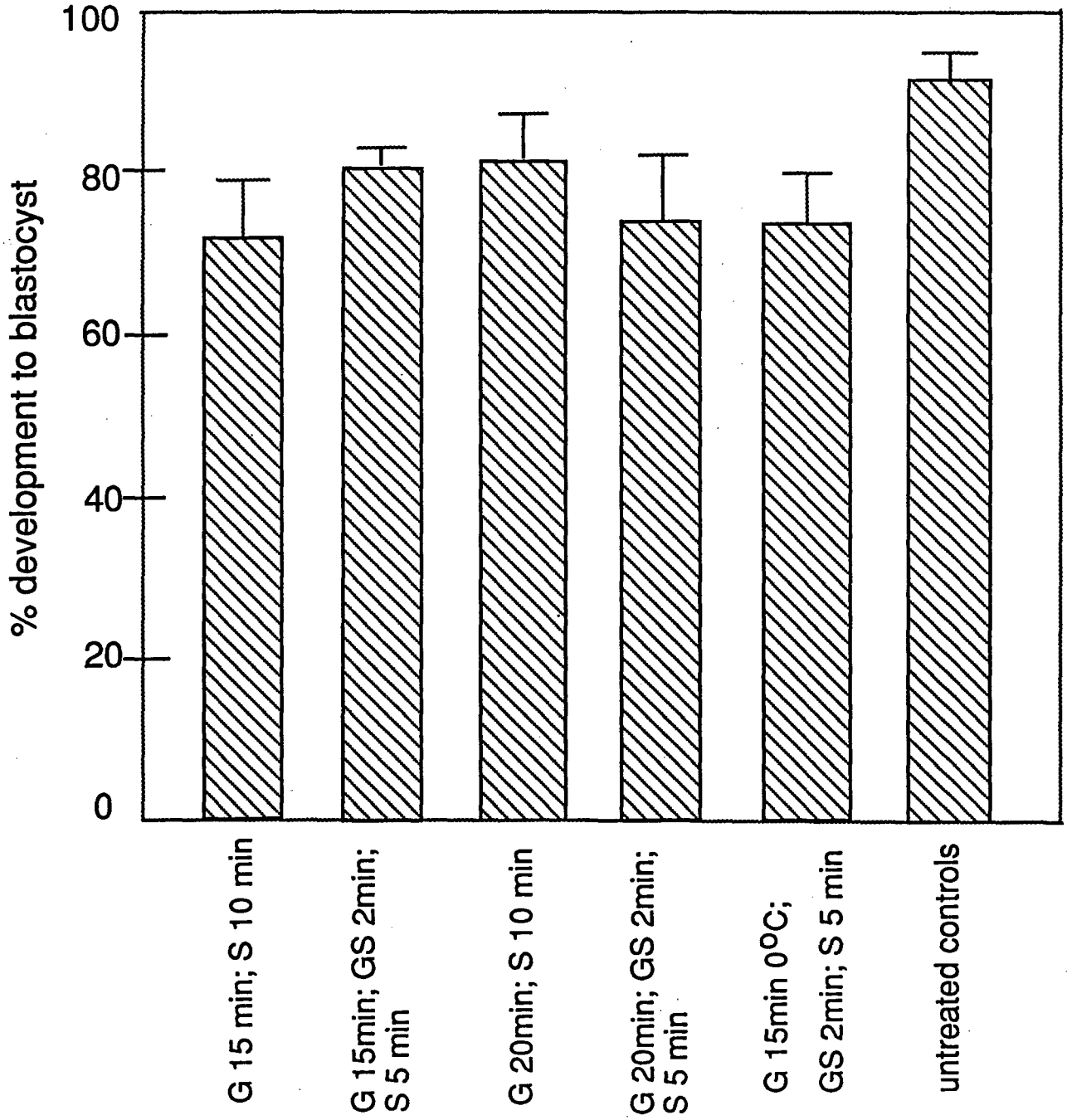
++Morulae collected from 10 superovulated donors. Not all embryos collected were used in these experiments.

*Viablity was assessed after 1-3 h in culture and percentages based on number of morulae frozen.

**Based on the number of embryos transferred into females that established pregnancy.

^{a,b,c}Values with different superscripts within each column indicate significant differences ($p<0.05$).

Figure 1: Effects of cryoprotectant, temperature and dehydration/rehydration regimen on rat morulae development in vitro.



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